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pH-dependent formation of ethylenediaminetetraacetic acid supramolecular aggregates

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Abstract

Pure ethylenediaminetetraacetic acid tetrasodium salt (EDTA · Na_4) in water showed a pH-dependent change of optical density detected in the ultraviolet region. The most important change was found between pH 4 and 8, corresponding to the deprotonation of the third ionizable group having a pK of 6.2. The absorbance change was reversible and depended on the addition/removal of only 1 proton equivalent. The presence of supramolecular aggregates was suggested by size-exclusion chromatography, dialysis and cryo-electronmicroscopy. The molecular weight of EDTA supramolecular aggregates was 10-15,000 and 8-14,000, as determined by gel chromatography and dialysis, respectively. Cryo-electronmicroscopy revealed structures of 2–4 nm in diameter.

Key words: Fibrin; γ-Chain-petide; Polymerization; Ethylenediaminetetraacetic acid; Supramolecular aggregate

1. Introduction

EDTA, a chelating agent, is widely used in biology, food technology, and the pharmaceutical industry to complex divalent cations, mainly calcium and other heavy metals. Researchers dealing with calcium-dependent regulation mechanisms (e.g. blood coagulation) and/or with proteins with structures and biological activity that are calcium-dependent, make use of EDTA and its parent molecule, EGTA, to buffer or to complex calcium. The use of these chelating agents is not free of problems because they may bind to proteins interfering with the expression of biological activity, e.g. fibrin polymerization [1–3]. Such problems strongly retarded the progress of the characterization of the complementary binding sites in fibrin involved in the polymerization process [4,5]. Fibrinogen contains at the N-termini four protected polymerization sites which are activated by thrombin, releasing two fibrinopeptides termed A and B. The new exposed N-termini react with complementary polymerization sites in the C-terminal region of a parent molecule thus starting the polymerization process. Characterization of complementary binding sites was undertaken by plasmin digestion of fibrinogen in the presence of calcium ions, resulting in the production of fragments E (N-terminal region) and D_1, which contains the complementary polymerization site [6]. Fragment D_1 added to activated fibrinogen (fibrin) molecules inhibited the polymerization process [6]. Further digestion of fragment D_1 by plasmin is obtained by complexing calcium with EDTA or EGTA, resulting in the production of fragments D_2 and D_3, which have lost their capability to inhibit fibrin polymerization [5]. Analysis of fragment D_3 showed that the former plasmin treatment selectively removed 109 amino acids of the γ-chain C-terminus, implying, therefore, that this region may be involved in the complementary binding sites. It was thought that peptides derived from the proteolysis were capable of inhibiting fibrin polymerization [7]. Synthetic peptides corresponding to the putative complementary binding site region were not able to inhibit polymerization [8]. The artifactual anti-polymerization activity was attributed to EDTA [5].

2. Materials and methods

2.1. Materials

EDTA · Na_4, EGTA were from Fluka (Buchs, Switzerland); HEPES, Tris were from Sigma (Buchs, Switzerland); fibrinogen was from Kabi Vitrum (Mölndal, Sweden). All other reagents were of analytical grade.

2.2. Fibrinogen fragments D_1 and D_3

Fragment D_1 was prepared by limited proteolysis of fibrinogen with plasmin and purified by affinity chromatography as previously described [9]. Further plasminolytic treatment of fragment D_1 was performed overnight at 37°C in the presence of 5 mM EDTA [10,11]. Fragment D_3 was separated from other smaller peptides by gel permeation chromatography on a Superose 12 10/30 column (Pharmacia, Uppsala, Sweden) with a FPLC pump system (Pharmacia) equipped

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Abbreviations: EDTA, Ethylenediaminetetraacetic acid; EGTA, Ethylene glycol-*O*,*O*′-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; M_r, molecular mass; Tris, tris(hydroxymethyl)aminomethane.

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with a Kratos Spectroflow 773 absorbance detector (Westwood, NJ, USA). Fibrin polymerization/anti-polymerization activity of fragments was measured with a coagulometer according to Schnitger & Gross (Amelung, Lemgo, Germany) and expressed as clotting time in seconds. SDS-PAGE of fibrinogen fragments was performed according to Laemmli [12].

2.3. EDTA

Spectral analysis was performed with a Kontron Uvikon 810 spectrophotometer (Zürich, Switzerland) and with a Hewlett Packard HP 8542A diode array spectrophotometer (Waldbronn, Germany). pH measurements were performed with a Metrohm pH meter E 603 equipped with a combined pH glass electrode (Herisau, Switzerland). Spectra/Por dialysis membranes with 3,500, 6–8,000, and 12–14,000 M_r cut-off were from Spectrum (Los Angeles, CA, USA).

2.4. Cryo-electron microscopy

Cryo-electronmicroscopy was performed as described by Adrian et al. [13].

3. Results

In order to elucidate the unexplained behavior of EDTA, fragment D_1 plasmin lysates were re-analyzed in moral detail.

Fig. 1A shows a chromatogram of a fragment D_1 plasmin lysate in the presence of 4 mM EDTA run on a Superose 12 column, which separated proteins by size. Absorbance at 280 nm (solid line) revealed the presence of protein material eluting after the main peak (fragment D_3) and having inhibitory activity against the polymerization of fibrin as assayed by clotting times (Fig. 1A, dotted bars). The approximate M_r of these active fractions was 10–15,000, as judged from calibration experiments with ribonuclease (M_r = 13,700; Stoke's radius = 1.75 nm). The M_r of γ-chain peptides produced by plasmin digestion were expected to be in the same range considering their maximal length of 109 amino acids. Experiments performed with larger amounts of fragment D_1 (about 100 mg) and chromatographed on Sephadex G-200 produced similar results (not shown).

Chromatography of 4 mM EDTA in 20 mM HEPES buffer, pH 7.4, on Superose 12 without any added protein under the same conditions as used for D_1 plasmin lysate separation, gave absorption peaks at 280 nm (Fig. 1B, solid line) at the same position where the fractions containing fibrin anti-polymerization activity were eluted (Fig. 1B, dotted bars), thus showing the presence of EDTA. The artifactual activity of putative inhibitory peptides derived from fragment D_1 was therefore caused by EDTA. This activity could be suppressed by the addition of 2.5 mM $MgCl_2$ to the reaction mixture before assay in the coagulometer (Fig. 1A,B, solid bars). Addition of 50 μM EDTA to inactive fractions of the chromatogram produced prolonged clotting times similar to those found in the fractions where EDTA was chromatographed with the protein mixture. These activities were suppressed by the addition of 500 μM $MgCl_2$ (not shown), confirming the suggestion that EDTA was no longer interfering with the fibrin polymerization process but was complexed by Mg^{2+} ions. The fact that a molecule like EDTA with a M_r of 292 eluted from gel permeation chromatography much earlier than expected and gave an absorption at 280 nm, was quite anomalous, suggesting that EDTA is able to form higher molecular weight structures in solution.

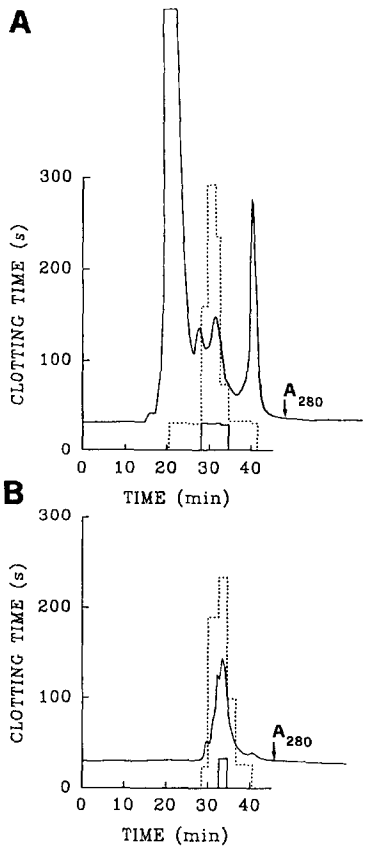

Fig. 1. Chromatography of fragment D_1 lysate, EDTA, and their fibrin anti-polymerization activity. (A) 0.5 ml 10 mg/ml fragment D_1 lysate in 100 mM NaCl, 50 mM Tris-Cl, 4 mM EDTA, pH 7.4, were chromatographed on a Superose 12 10/30 column, flow rate 0.5 ml/min, in 20 mM Tris-Cl, pH 8.2, at room temperature, and protein was detected at 280 nm (solid line). Fractions (50 μl) were analyzed for their fibrin anti-polymerization activity. The main peak corresponded to fragment D_3, which had no fibrin anti-polymerization activity (dotted bars). Fractions eluted after fragment D_3 showed a clear prolongation of the fibrin clotting time. This activity was reduced to control values by the addition of 2.5 mM $MgCl_2$ to the reaction mixture in the coagulometer (solid bars). Most probably this effect was due to complex formation between EDTA present in the sample and Mg^{2+} ions. (B) In analogy to A, 0.5 ml 4 mM EDTA in 20 mM HEPES-NaOH, pH 7.4, were chromatographed under identical conditions. Absorption peaks appeared at about the same elution volumes as found in the chromatogram of fragment D_1 lysate. Fractions showed fibrin anti-polymerization activity which was suppressed by the addition of $MgCl_2$.

Fig. 2a shows that EDTA in water produces a conspicuous change in its absorption as a function of pH (curve A basic conditions, curve B acidic conditions). Titration of 10 mM EDTA tetrasodium salt (pH 11; A_{240} = 2.8) with HCl (Fig. 2b) showed that: (i) the addition of the

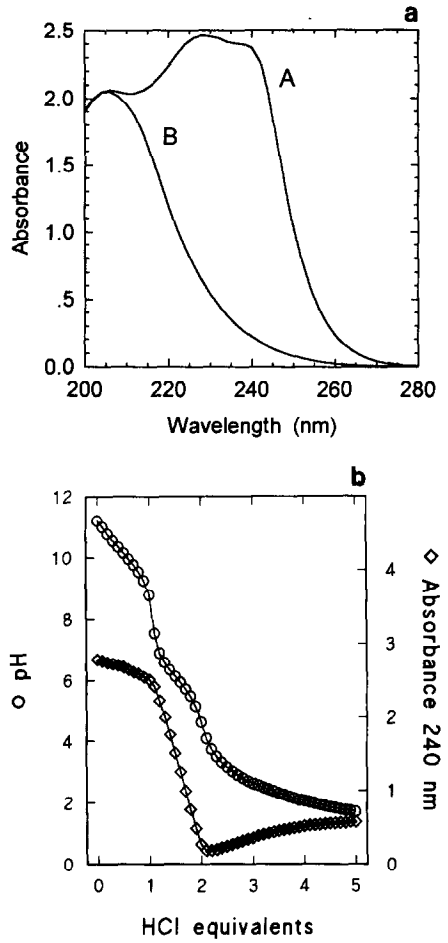

Fig. 2. pH-dependent spectral changes and titration of a 10 mM EDTA solution. (a) Curve A: 10 mM EDTA tetrasodium salt dissolved in water had a pH of 11. Curve B: addition of concentrated HCl reduced substantially the absorption of the solution. (b) 100 ml of the same solution used in (a) was titrated with 1 M HCl. Samples were analyzed for their pH (○) and absorption at 240 nm (◇). The addition of the second HCl equivalent produced the most evident variation in absorption. It corresponds to the titration of the second protonable imidodiacetic group of EDTA, having a pK of 6.2.

first proton equivalent (pK = 10.3) produces a flat diminution of the absorption intensity (pH 8; A_{240} = 2.45); (ii) the addition of the second equivalent (pK = 6.2) shows a steep decrease of the absorption intensity, reaching a minimum value (pH 4; A_{240} = 0.2); (iii) the addition of another three equivalents (pK = 2.7 and 2.0) leads to a continuous increase in absorption with much lower intensity as compared to the starting condition (pH 2; A_{240} = 0.55). Further addition of HCl precipitated EDTA as free acid. The sharp change in absorption correlated with the change in pH by the titration of the second protonable group with a pK of 6.2. The introduction of an additional positive charge on the EDTA molecule generated the molecular species with maximal number of charges (two positive and four negative), and therefore with the maximal solubility, as revealed by the minimum in absorption at pH 4. Removal of one positive charge going from pH 4 to 8.7 was sufficient to induce a large absorption change which may be interpreted as formation of EDTA supramolecular aggregates.

The presence of such structures was analyzed by dialysis experiments. Fig. 3a shows that using a membrane with a M_r cut-off of 3,500, EDTA dialyses at a much slower rate at alkaline pH compared to acidic pH, and in any case slower than ferricyanide (M_r = 212), which

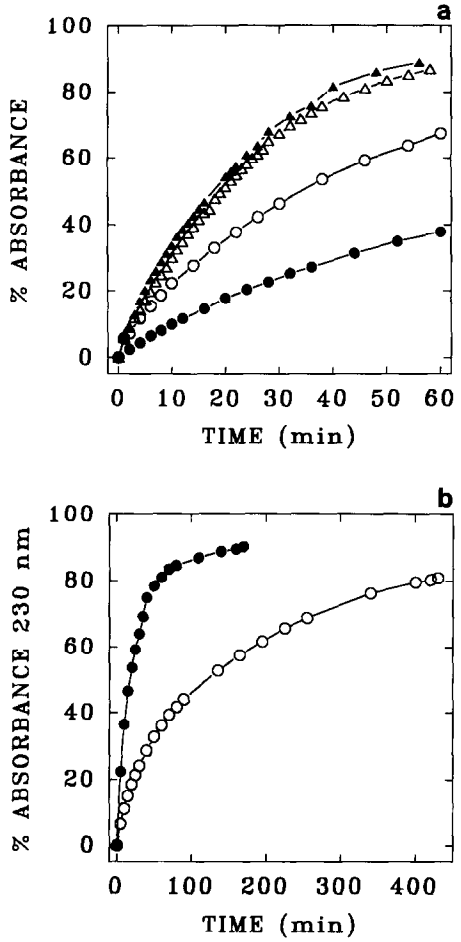

Fig. 3. Dialysis of EDTA solution. (a) 1 ml 10 mM EDTA, pH ≅ 11, was dialized against 100 ml of 5 mM NaOH, pH ≅ 11, through a Spectrapor membrane with a M_r cut-off of 3,500 (●); for dialysis at acidic pH, EDTA was initially titrated down to pH ≅ 4 with 2 HCl equivalents and dialyzed against 1 mM acetic acid, pH ≅ 4 (○); for comparison 1 ml 1 M ferricyanide was dialyzed under similar conditions at basic (△) and acid (▲) pH. Both EDTA and ferricyanide were measured spectroscopically in the outer compartment at 220 and 450/460 nm, respectively. Although both substances have similar M_r's EDTA clearly dialyzed at a slower rate than ferricyanide. Ferricyanide did not show a significant difference between dialysis at acidic or at basic pH, whereas EDTA dialysis at basic pH was slower than at acidic pH. This observation is in agreement with the idea that EDTA in solution forms structures the size of which is pH-dependent. (b) EDTA was dialyzed as described in (a) under basic conditions but with 6–8,000 (○) and 12–14,000 (●) M_r cut-off dialysis membranes, respectively. EDTA supramolecular aggregates dialyzed through the 6–8,000 membrane as slowly as shown in (a) for the 3,500 membrane, whereas the 12–14,000 membrane did not retain the EDTA supramolecular aggregates. It is therefore concluded that the M_r of EDTA supramolecular aggregates is in the range between 8 and 14,000.

does not show a pH dependence of its dialysis velocity. To explain the fact that EDTA supramolecular aggregates could be dialyzed, it must be assumed that such structures are in a dynamic equilibrium with single molecules in solution which can pass through the membrane pores without resistance due to their low M_r. This situation seems to be similar to the behavior of detergent or lipid molecules forming micelles. The larger sized aggregates are unable to pass through the membrane pores whereas monomer molecules can. The size of EDTA supramolecular aggregates was measured using dialysis membranes with different M_r cut-offs. Fig. 3b shows that membranes with a M_r cut-off of 6–8,000 still retarded EDTA dialysis, whereas a 12–14,000 M_r cut-off membrane allowed free dialysis of EDTA supramolecular aggregates. Thus, the M_r of supramolecular aggregates determined by this technique was between 8,000 and 14,000, which is in good agreement with results obtained by gel permeation chromatography. Dialysis experiments carried out with 12–14,000 M_r cut-off membranes

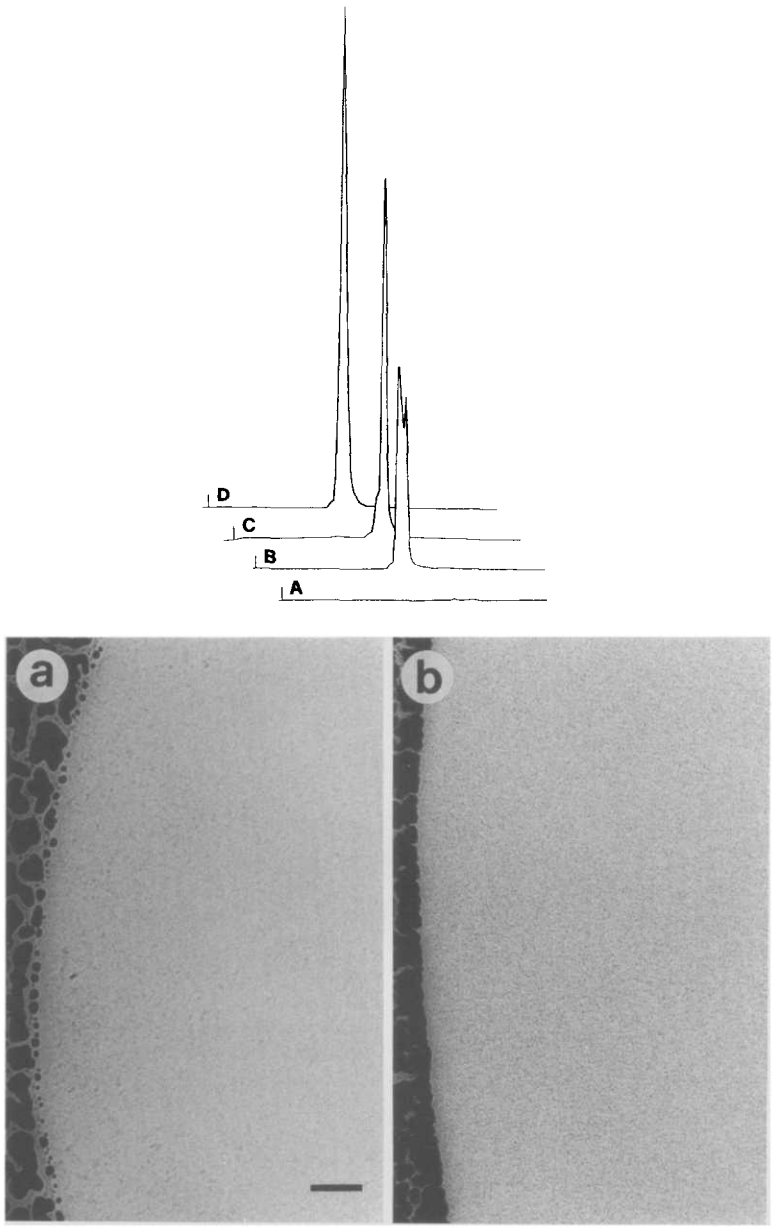

Fig. 4. Visualization of EDTA supramolecular aggregates. (Upper panel) Chromatography of EDTA and its complex with lead acetate on Superose 12 in 20 mM HEPES-NaOH, pH 7.4, absorption detection at 260 nm, sample 0.5 ml. Trace A, 1 mM Pb $(CH_3COO)_2$, 20 mM NaOH; B; 4 mM EDTA, 20 mM NaOH; C, 4 mM EDTA, 20 mM NaOH, 1 mM $Pb(CH_3COO)_2$; D, 4 mM EDTA, 20 mM NaOH, 2 mM $Pb(CH_3COO)_2$. Traces C and D show that the addition of lead acetate to EDTA solution at basic pH increased its absorption, possibly by increasing the size of the supramolecular aggregates. (Lower panel) Cryo-electron micrographs of 4 mM EDTA, 20 mM NaOH, 1 mM $Pb(CH_3COO)_2$ at basic (a) and acidic (b) pH. At acidic pH the frozen solution did not present any kind of visible structure, whereas at basic pH circular structures with a diameter of 2–4 nm were visible. Structures accumulate due to freezing at the edge of frozen droplets. Bar = 40 nm.

using a dialysis buffer composed of 20 mM Tris or HEPES, both pH 7.4, did not show any significant difference from the experiments performed at basic pH (not shown).

Further support for the existence of EDTA supramolecular aggregates was obtained by analyzing pictures of EDTA–lead acetate complexes photographed in a cryo-electron microscope. Particles with a radius of 2–4 nm were visible in samples frozen and photographed at alkaline pH (Fig. 4a, lower panel), whereas it was not the case at acidic pH (Fig. 4b, lower panel). Interestingly, at basic pH, EDTA supramolecular aggregates can still complex ions, as shown in this case by lead acetate. Addition of lead acetate to EDTA solution (Fig. 4, upper panel) increased and standardized the peak fraction eluting from the gel permeation column, indicating an interaction between EDTA and lead ions possibly associated with an increase in the structure size.

In conclusion, it was possible to present evidence supporting the idea that EDTA can form supramolecular aggregates. These EDTA supramolecular structures, the formation of which is pH dependent in the physiological range, may possibly contribute to artifactual phenomena in biological systems on the one hand, whilst on the other hand their peculiar characteristics may be usefully exploided.

4. Discussion

At first sight it was inconceivable that EDTA might possess properties other than those for chelating metal ions. The experimental evidence presented in this work (UV-spectroscopy, gel permeation chromatography, dialysis, and cryo-electron microscopy) suggests however, that EDTA is able to change its aggregation state as a function of pH. At pH 4, EDTA has the maximal number of charges; four negative from the carboxylates, and two positive ones localized on the imino moieties. These charges confer to the molecule the maximal solubility and the minimal light absorption. Removal of one proton from the molecule induced an evident increase in light absorption, which was interpreted as formation of aggregates. The deprotonation of one of the two iminodiacetates (pK = 6.2) may have created an hydrophobic micro-environment which would favor the aggregation. The effect became more evident when the second proton located on the second iminodiacetate was removed (pK = 10.3). At basic pH the molecule was strongly polarized, having on each edge two negative charges separated by an uncharged and apolar region. This description resembles the concept of the formation of a lipid bilayer as well as that of micelles. These supramolecular structures were thus present at commonly used pH's, and therefore may be the source of problems or artifacts in concomitance with biological material. An example of interference was reported in Fig. 1: the unexpected presence of EDTA after gel permeation in samples containing peptides led to the wrong conclusion that the peptides had inhibitory activity against fibrin polymerization, whereas the effect was, in fact, due to the EDTA. If the biological system under study is sensitive to divalent cations and/or to chelators, it is imperative to ascertain that there is no interference by the chelator. The same attention should be given to dialysis experiments using EDTA solutions even under physiological buffer conditions where EDTA has not apparently been creating any problems; unless, however, there is an interaction between a protein and EDTA itself.

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